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Award Number: W81XWH-10-1-0927

TITLE: Mesenchymal Stem Cell Therapy for Nerve Regeneration and Immunomodulation after Composite Tissue Allotransplantation

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REPORT DATE: February 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-02-2011	Annual	20 Sep 2011 - 31 Jan 2012

4. TITLE AND SUBTITLE
Mesenchymal Stem Cell Therapy for Nerve Regeneration and Immunomodulation after Composite Tissue Allotransplantation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0927

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Johns Hopkins University
Baltimore, MD 21218

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Composite Tissue Allotransplantation (CTA) offers an alternative reconstructive strategy for complicated musculoskeletal injuries incurred during modern warfare where functional outcomes after multiple conventional reconstructions are suboptimal. Broader clinical application of CTA, however, continues to be hampered by requirement for long-term multi-drug immunosuppression to prevent graft rejection. Furthermore, unlike in solid organs, clinical success is dictated not only by graft acceptance, but also by functional outcome. Our study proposes a novel cell-based therapy utilizing mesenchymal stem cells (MSC) that can augment nerve regeneration while minimizing the need for immunosuppression. After transition from University of Pittsburgh to Johns Hopkins University, we optimized our isolation and culture protocol for MSCs. Immunophenotypic and functional characterization of cultured cells demonstrated potent immunomodulatory effects of MSCs in vitro. Preliminary functional outcome analysis using Catwalk showed convergent data with similar loss and return of function among groups. Similarly, compound muscle action potentials at early time points showed small non-significant increases in amplitude in experimental groups. In contrast, normalized gastrocnemius weights showed increased muscle weight in only the systemic injection group. Overall, we continue to monitor both functional and histological outcomes. Consistent with existing literature on nerve regeneration, we expect to observe more evident differences among groups with increased data over the longer term.

15. SUBJECT TERMS
Composite tissue allotransplantation, Mesenchymal stem cell therapy, Nerve regeneration, Immunomodulation, Hind-limb, Sciatic nerve

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	16	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Composite Tissue Allotransplantation (CTA) is an innovative reconstructive modality that can provide functional restoration after complex musculoskeletal trauma such as upper extremity amputation or disfiguring facial trauma[1]. CTA has provided new hope for service members suffering from catastrophic combat trauma. The implementation of cellular therapies that integrate the concepts of immune regulation with those of nerve regeneration can optimize the functional outcomes of these reconstructive modalities and minimize the need of immunosuppression. Bone Marrow Derived Mesenchymal stem cells (BM-MSCs) are pluripotent cells, capable of differentiation along multiple mesenchymal lineages into osteocytes, chondrocytes, myocytes, and adipocytes[2]. Recent advances have shown that MSC can also trans-differentiate into Schwann cells (SC) [3]. MSC-enhanced nerve regeneration has been demonstrated both *in vitro* and *in vivo*. Other than bone marrow, MSCs are also present in adipose tissue, skin, heart and placenta and can be isolated and expanded *ex vivo* thereby emerging as promising tool for cell based therapeutic strategy[4]. Recently, BM-derived MSC have been identified to have potent immunosuppressive properties. Most importantly, MSCs are considered to be immunoprivileged by their low immunophenotype[4-5]. MSC offer some potential advantages over conventional immunosuppressive agents by specifically targeting immunoinhibitory effects that could prevent rejection, and minimize the systemic complications of nonspecific immunosuppressant in the setting of CTA[6-7]. In addition to the immunomodulatory effects of MSC, they have demonstrated the ability to prevent and treat GVHD[8], one of the most serious complications following transplantation. Using our proposed models, in the present study we are investigating combined neuroregenerative and immunomodulatory effect of BM-MSCs following systemic administration.

PROGRESS OF ONGOING PROJECT AND RESEARCH ACCOMPLISHMENTS

TASK 1: Demonstrate neuroregenerative effect of systemic MSC in a sciatic nerve transection model.

As part of the implemented transition plan of CTA laboratory from the University of Pittsburgh to Johns Hopkins University, a new ACUC protocol was submitted and approved by Johns Hopkins University Animal Care and Use Committee.
The transition from the University of Pittsburgh to Johns Hopkins University required significant efforts to optimize surgical technique, cell culturing methodology, electrophysiology measurement, and histological preparation. After several months of recursive optimization and data quality evaluation, the first task was undertaken in a sciatic nerve transection model. The following paragraphs describe the optimized protocols and early findings.

MILESTONE 1A: Establish MSC Culture Protocol.

BM-MSC HARVEST:
Bone marrow derived MSCs were harvested from femur and tibia of anesthetized adult Brown Norway rats (4-6 weeks old). BM-MSCs were isolated based on their inherent plastic adherence when grown in culture media. After 2-3 passages, MSCs were further purified via FACS sorting.

IMMUNOPHENOTYPIC AND FUNCTIONAL CHARACTERIZATION:
Immunophenotypic characterization was done by flow cytometry analysis. MSCs consistently and homogenously expressed CD29 and CD90 and were negative for CD11, CD45, RT1A and RT1B (Figure 1).

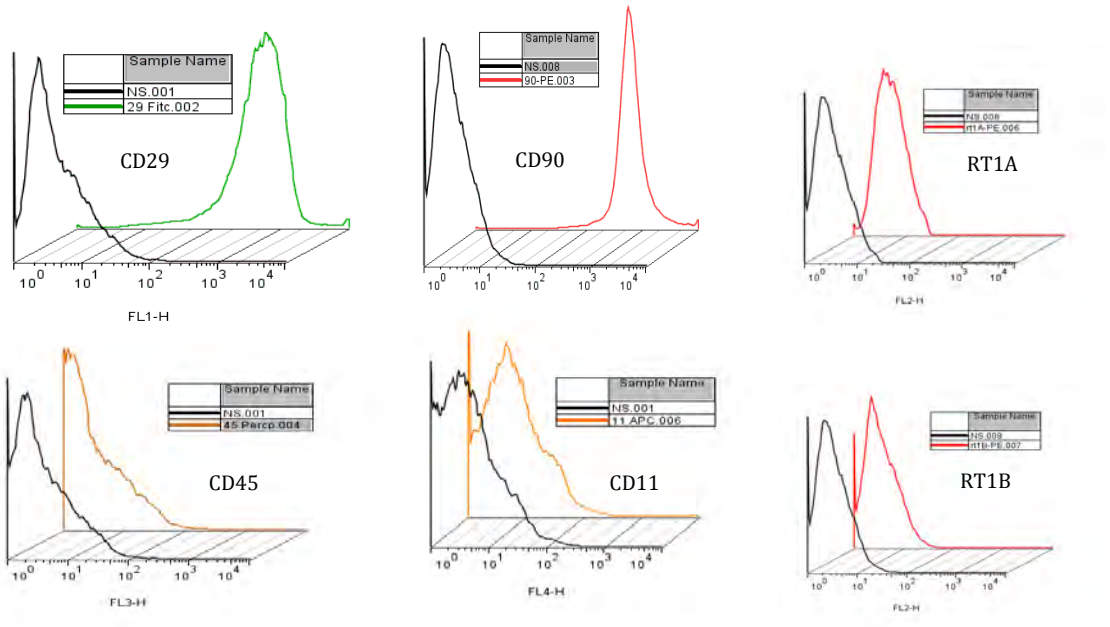

Figure 1: BM-MSCs consistently and homogenously expressed cell surface markers CD29 and CD90 and were negative for CD45, CD11, RT1A and RT1B as determined by flow cytometry analysis using fluorescent labeled monoclonal antibodies.

The culture-grown BM-MSCs were tested for their ability to differentiate into adipocytes, osteoblasts, and chondrocytes: Osteoblasts were identified by von Kossa staining, adipocytes by oil-red O staining, and chondrocytes by Alcian blue staining (Figure 2).

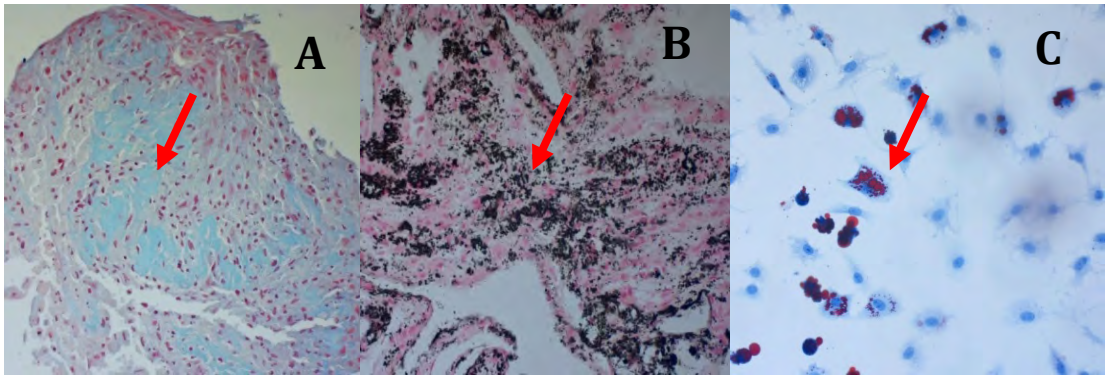

Figure 2: Differentiation Potential of BM-MSCs: (A) Chondrogenesis: Arrow indicated Blue-Stained deposition with Alcian-Blue, suggesting the formation of collagen,
(B) Osteogenesis: With Von Kossa Staining, the cytoplasmic calcium deposition was shown as black deposition (Arrow) and (C) Adipogenesis: Oil-O Red staining showed the intracellular lipid-filled vesicles (Arrow).

MILESTONE 1B: Complete sciatic nerve transection procedures.

Figure 3: Schedule of surgeries. Each group includes 4 animals. The first three groups have ongoing data capture. The lower three groups were sacrificed for 6 week data.

SCIATIC NERVE TRANSECTION AND REPAIR:

As detailed in the original statement of work, eight animals in one of three groups received sciatic nerve transections with repair. As part of the "control" group, eight animals received sciatic nerve transection with suture repair. No cells were injected in this group. In the "Local" and "Systemic" groups, eight animals each received sciatic nerve transection with either local administration of MSCs into the distal stump or a systemic injection of MSCs, respectively. The three groups of eight were then further subdivided into a 6 week and 12 week measurement group. The surgical schedule is shown in Figure 3.

All Sciatic Nerve Transection and Repair procedures were performed in a similar fashion. A gluteal skin incision was made from the area of sciatic notch to just above the knee joint. The gluteal muscles were separated in order to expose the Sciatic nerve from the sciatic notch to

the point of bifurcation. Then, the nerve was transected and approximated with interrupted epineurial 10-0 nylon suture (Figure 4). All animals in the local injection groups received 50,000 MSC injected into the distal nerve stump using a 33-gauge needle attached to a custom made 50-µl syringe. All animals in the systemic groups received one million MSCs injected into tail vein at the end of procedure.

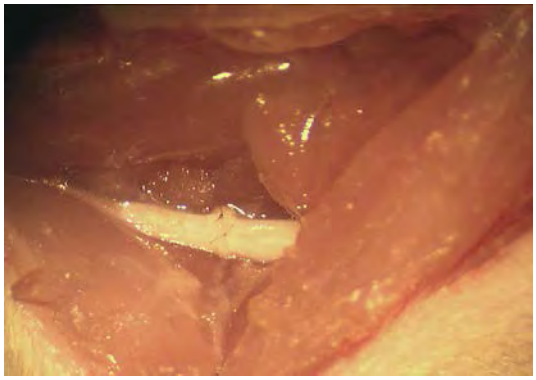

Figure 4. Sciatic nerve transected and repaired primarily using 10-0 epineureal sutures.

HIND LIMB TRANSPLANT:

We optimized our orthotopic VCA hind limb from a Lewis-to-Lewis rat (syngeneic transplant) or Brown Norway to Lewis rat (allogeneic transplant, Figure 5). In brief, the femoral nerve, artery and vein were isolated and divided ensuring adequate length for subsequent anastomoses. The remaining thigh muscle groups as well as the sciatic nerve were transected to expose the mid-portion of the femur. A transverse osteotomy was performed through the femur to complete allograft harvest. The recipient animal was prepared in a similar fashion. Transplantation of the allograft was performed with osteosynthesis of the femur. The femoral vein and then femoral artery were anastomosed. The sciatic as well as the femoral nerve were approximated with interrupted epineurial 10-0 nylon sutures. The ventral and dorsal muscle groups were then repaired with 4-0 Vicryl prior to skin closure with 4-0 Ethilon. Cell delivery method will remain the same as described for Sciatic Nerve Injury Model.

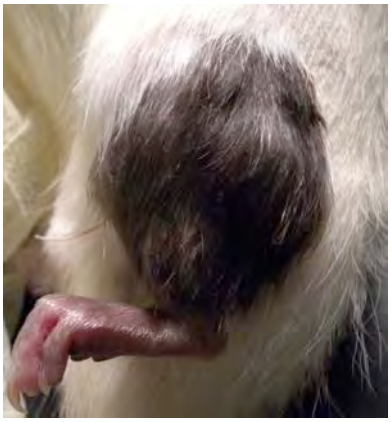

Figure 5: Orthotopic hind limb transplant from Brown Norway to Lewis Rat

COMPUTER-ASSISTED GAIT ANALYSIS USING CATWALK:

Catwalk XT System (Noldus Technology). The CatWalk XT provides advanced gait analysis in rodents. The system consists of an enclosed walkway, a high-speed color camera, and recording and analysis software to assess the locomotor performance of rodent models. While animals traverse the walkway from one side to the other side in a non-enforced manner, their footprints are captured with a high-speed video camera. The video camera sends the capture to a computer that runs the CatWalk XT software. Utilizing Illuminated Footprint technology, the paw print area, contact intensity, swing speed and swing distance are captured. From this data, numerous parameters are calculated for qualitative and quantitative analysis of individual footfalls and gait. In order to provide consistent and reproducible functional outcome data, we introduced pre-operative training of the rats on the CatWalk system. As a result, their walk through the CatWalk walkway became unforced, continuous and consistent, leading to greater accuracy in the classification of their walking behavior. In addition, the calibration settings of the CatWalk were optimized to increase the detection accuracy of the machine.

Pilot experiments were conducted involving various models of rat peripheral nerve injuries (sham, nerve gap, crush injury, transection and repair) to confirm reproducibility of data. After optimization, we recorded weekly functional outcome data on the three sciatic

transection and MSC injection experimental groups. A selection of this data is shown in Figure 6. Maximum paw intensity vs. time is shown in Figure 6 A-B. Maximum paw intensity remained consistent in the un-injured contralateral limb whereas gradual recovery was

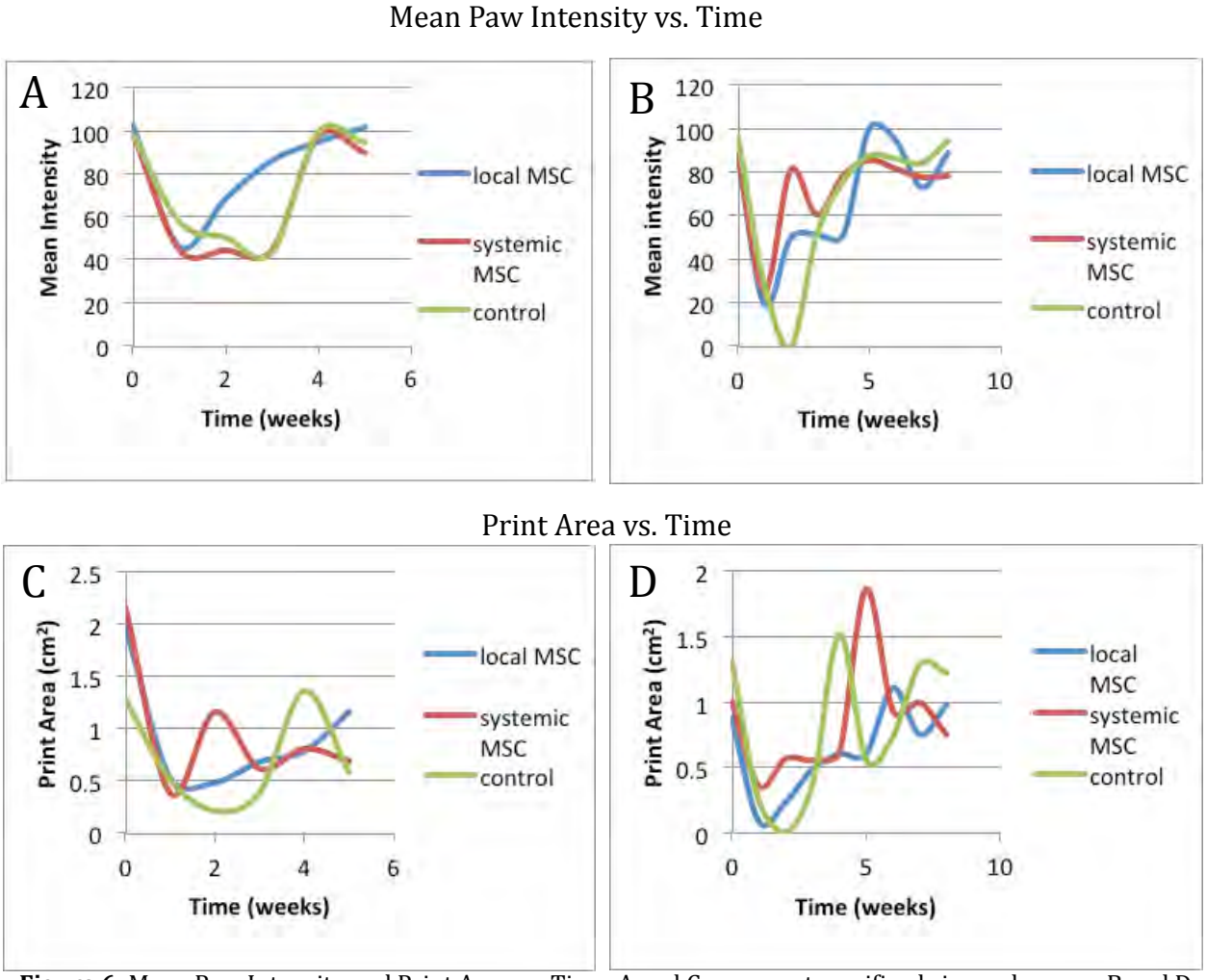

Figure 6: Mean Paw Intensity and Print Area vs. Time. A and C represent sacrificed six week group. B and D represent long-term group with 8 weeks of functional data. Shown are the varying—and convergent—results among experimental groups.

observed in sham, crush injury and transection and repair. Nerve gap pilot group (transection without repair) showed minimal recovery (data not shown). Duty cycle, swing speed, base of support, print area and stride length were also found to be consistent in un-injured limbs. Figure 6 C-D shows print area vs. time. Weekly measurements on two groups of rats (six week group and twelve week group) show a similar behavior for all groups--with minor temporal deviations; all animals lose and regain functionality at similar time points. However, the local MSC group shows a promising difference in behavior; loss and gain of functionality is ultimately the same, but the pathway to these endpoints are reached more quickly. We continue to follow the animal data to see if this curious difference has any long-term effects.

ELECTROPHYSIOLOGY:

Using our new ADInstrument electrophysiology system, we optimized our compound muscle action potential (CMAP) recordings (Figure 7). The CMAPs were measured in the intrinsic foot muscles on the plantar surface using sub-dermal needle electrodes. Serial CMAP measurements are being performed on weekly basis on our experimental animals. The un-operated, contralateral side serves as the intrinsic control for the maximum attainable CMAP in a given rat.

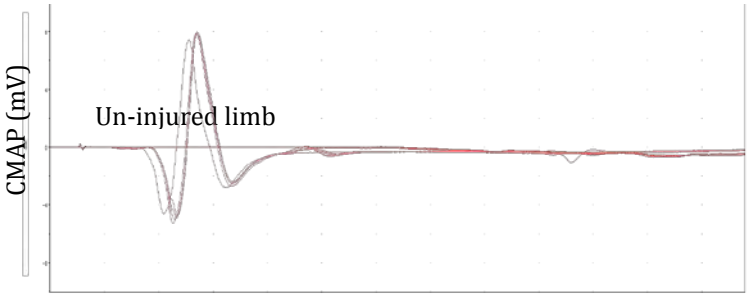

Figure 7: CMAP (mV) recordings from un-injured limb. Stimulating electrodes were placed near Sciatic notch and recordings electrodes were placed in intrinsic muscles of footpad.

Preliminary recorded maximal amplitude and latency data normalized to the contralateral, non-operated, limb is shown in Figure 8. Data is shown for changes in normalized EMG data from 2 and 5 weeks after nerve transection. Amplitude was measured as the maximal deflection from baseline, and latency was measured as the time from stimulation to this maximal deflection point. As expected, values for the experimental groups shortly after transection were low. Control transection and local injection groups showed a small non-significant increase in amplitude over time. A small drop was seen in the systemic injection group (Figure 8A). When adjusting for an outlier data point at these early recordings—which has now normalized in later recordings (not shown)—data for the groups was more in line with expected results (Figure 8B). Finally, Figure 8C shows normalized action potential

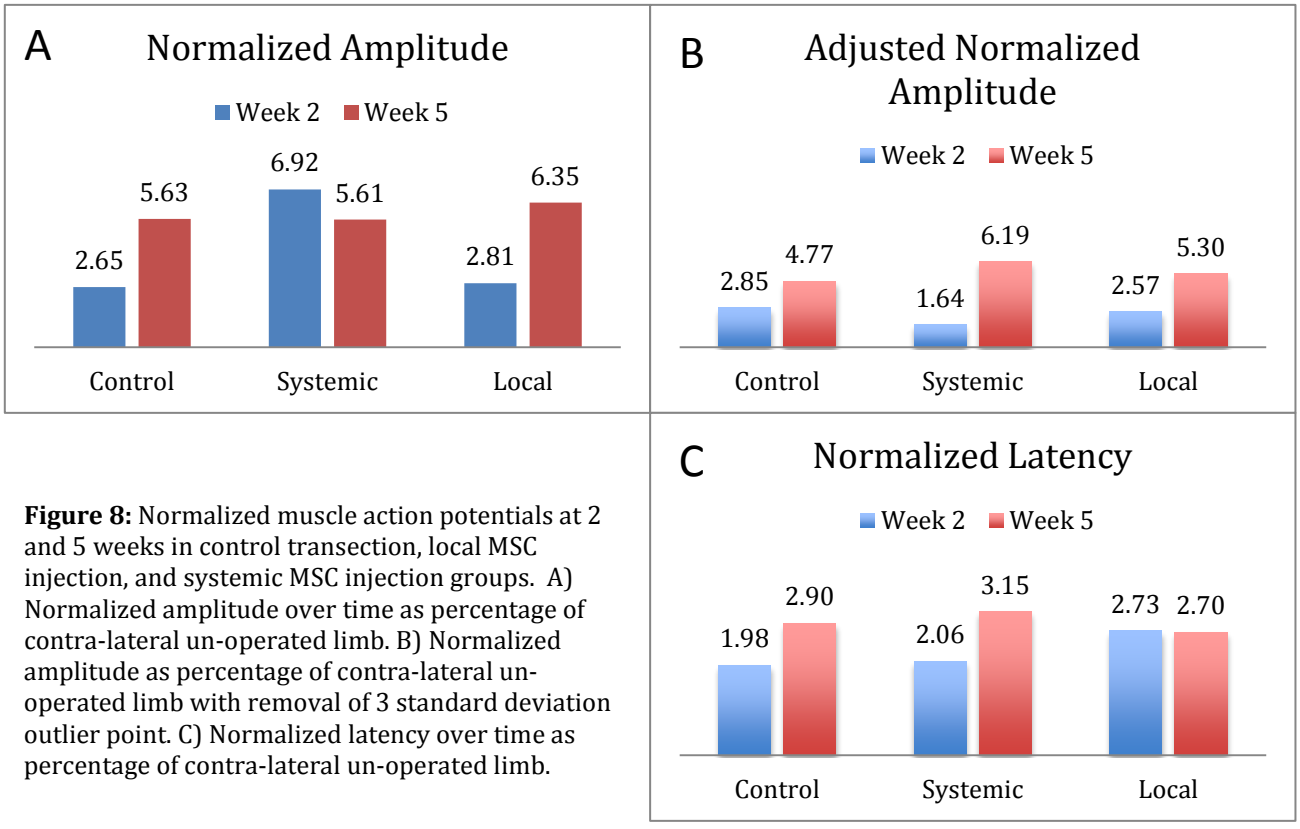

Figure 8: Normalized muscle action potentials at 2 and 5 weeks in control transection, local MSC injection, and systemic MSC injection groups. A) Normalized amplitude over time as percentage of contra-lateral un-operated limb. B) Normalized amplitude as percentage of contra-lateral un-operated limb with removal of 3 standard deviation outlier point. C) Normalized latency over time as percentage of contra-lateral un-operated limb.

latencies over time. At these first time points, no clear trend can be derived from the small

measured maximal amplitudes. At this early stage, it is not surprising that significant differentiation is seen between groups. We are closely monitoring our experimental group data for improvements in multiple factors up to 12 weeks after injury.

Nerve Gross Inspection and Histomorphometry :

Nerve and muscle was collected from the first data collection group sacrificed at six weeks. Gastrocnemius weights were measured for un-operated contralateral limbs and for the experimental limb in all groups. Results are shown in Figure 9 with normalization of experimental limb gastrocnemius weight to contralateral un-operated limb. Early data seems to indicate that the systemic MSC injection group may trend toward an increase in muscle weight—a proxy of improved nerve regeneration or reduced atrophy.

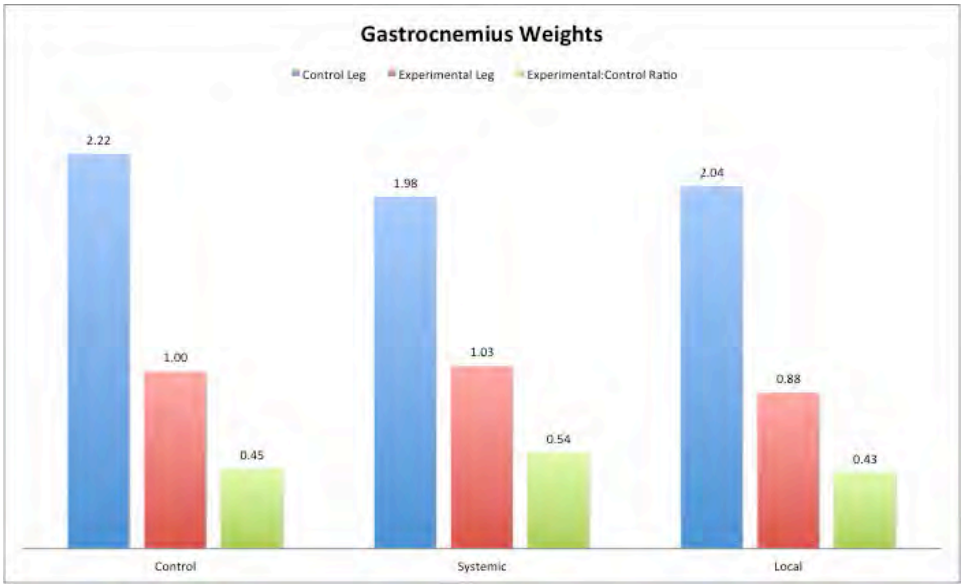

Figure 9: Gastrocnemius muscle weights for each experimental group at six weeks normalized to the un-operated leg.

In our newly established CTA lab, we optimized histomorphometric analysis of nerve sections for objective measure of axonal regeneration. Briefly, nerve section 5-8 mm distal to anastomosis site was fixed in glutaraldehyde, post-fixed with osmium tetroxide, and embedded in resin. Five micrometer thick cross-sections were cut and stained with toluidine blue for examination using light microscopy. Figure 10 shows representative optimized stains from our pilot groups. Nerve sections from the six-week data group have been fixed and prepared for sectioning. When sections have been prepared, six random high magnification fields from each nerve section will be analyzed using a digital image-analysis system linked to morphometric software (Image-Pro Plus, Bethedsda, MD). Using binary image analysis, we will measure the myelinated fiber count, nerve cross-sectional area, myelin thickness, axonal diameter, and fiber diameter.

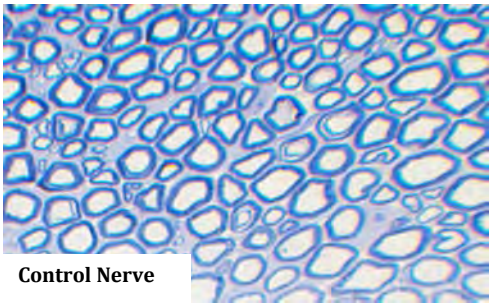

Figure 10: Transverse nerve section stained with toluidine blue for histomorphometric analysis, 40x magnification.

The measurements will be used to calculate fiber density, and average axonal and fiber area.

Effect of local vs. systemic MSC therapy:

Sciatic nerve injury and repair procedures/MSC therapy have been completed and animals are being followed with weekly functional outcome analysis as outlined in revised Statement of Work (Table 1)

Task 2 and 3: Determine immunomodulstory and neurogenerative effects of MSC therapy in Syngeneic and Allogeneic Hind Limb Transplant

Prior to performing hind limb transplant surgeries; we optimized our *in vitro* Mixed Lymphocyte Reaction (MLR) assays for determining immunogenicity and immunomodulatory pr operties of MSCs. Syngeneic and allogeneic hind limb transplant procedures will be performed as outlined in revised Statement of Work (Table 1).

In Vitro Immunogenicity and Immunomodulatory Properties of MSCs:

In Mixed Lymphocyte Reaction, bone marrow derived MSCs displayed very low immunogenicity. When compared with stimulated cells alone, co-culture of responding Splenocytes with BM-MSCs suppressed allogeneic stimulation (Figure 11).

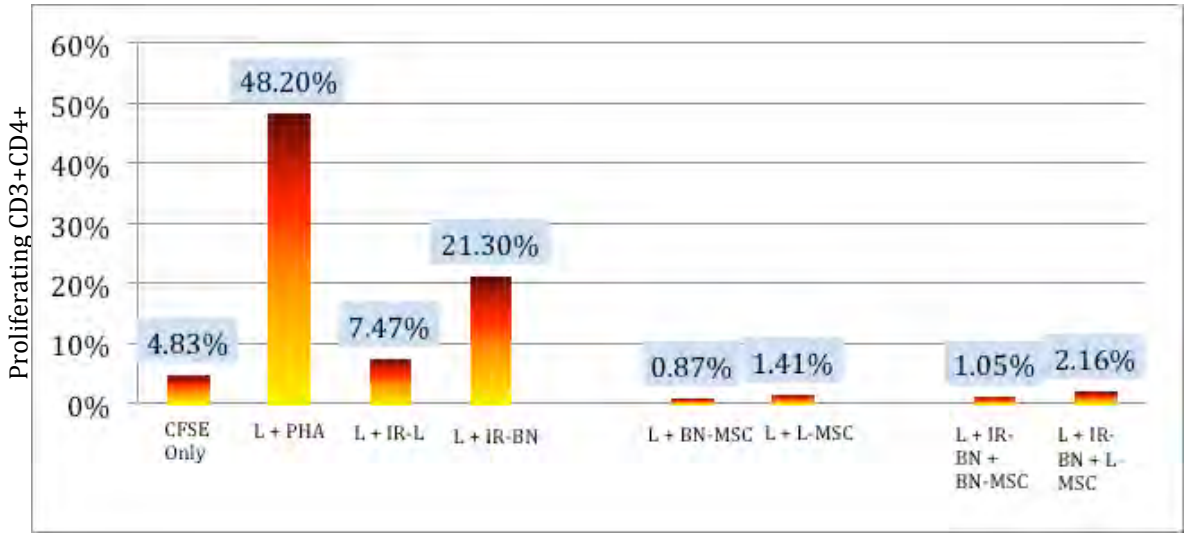

Figure 11: CFSE-based Mixed Lymphocyte Reaction: Co-culture of BM-MSC with responding Lewis Splenocytes suppressed allogeneic stimulation. (CFSE Carboxyfluorescein succinimidyl ester; PHA Phytohemagluttinin; L Lewis Splenocytes; IR-L Irridiated Lewis Splenocytes; BN Brown Norway Splenocytes; IR-BN Irradiated Brown Norway Splenocytes IR-BN; L-MSC Lewis BM-MSC; BN-MSC Brown Norway BM-MSC)

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of MSC isolation and culture protocol
- Establishment of mixed lymphocyte reaction to test immunogenicity and immunomodulatory properties of MSCs
- Optimization of surgical techniques for sciatic nerve repair, rat hind limb allotransplantation and cell delivery methods
- Optimization of catwalk system for video assisted gait based functional analysis
- Optimization of electrophysiological techniques for quantitative assessment of muscle re-innervation
- Optimization of histological techniques for quantitative assessment of axonal regeneration

CONCLUSION

As part of implemented transition from University of Pittsburgh to Johns Hopkins University, we optimized our mesenchymal stem cell (MSC) isolation and culture protocols, surgical techniques for our experimental models and modalities for functional outcome analysis. We are in the process of monitoring functional outcome of sciatic nerve repairs utilizing MSCs and will investigate the role of MSCs in nerve regeneration and immunomodulation as outlined in the revised Statement of Work.

A request for a six-month no-cost extension has been submitted to allow us to complete the remaining experiments as outlined in the revised SOW (see below)

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REVISED STATEMENT OF WORK AND REQUEST FOR NO COST EXTENSION:

Table 1: REVISED STATEMENT OF WORK

	1ST YEAR	EXTENDED MONTHS 1-3 (MARCH-MAY 2012)	EXTENDED MONTHS 4-6 (JUNE-AUGUST 2012)
SPECIFIC AIM 1 - Systemic MSC therapy improves regeneration after peripheral nerve injury.			
TASK 1 – Demonstrate neuroregenerative effect of systemic MSC in a sciatic nerve transection model.			
MILESTONE 1A – Establish MSC culture protocol	Johns Hopkins University ***(Completed)***		
MILESTONE 1B – Complete sciatic nerve transection procedures	Group I-III (Control, local MSC administration, systemic MSC administration) ***(In Progress)***		
DELIVERABLE 1 – Improved nerve regeneration by local/systemic treatment with purified *in vitro* cultured MSC	Sequential Catwalk, electrophysiology and histomorphometry ***(In Progress)***		
SPECIFIC AIM 2 - MSC therapy improves nerve regeneration following syngeneic composite tissue transplantation.			
TASK 2 – Determine neuroregenerative effect of systemic MSC therapy in a syngeneic CTA model.			
MILESTONE 2 – Complete syngeneic hindlimb transplants		Group IV (syngeneic hindlimb transplant) Group V (syngeneic hindlimb transplant + MSC)	
DELIVERABLE 2 – MSC-induced improvement of nerve regeneration in a syngeneic CTA model		Sequential Catwalk, electrophysiology and histomorphometry	
SPECIFIC AIM 3 - MSC therapy improves nerve regeneration and reduces the amount of immunosuppression required to enable graft survival in an allogeneic CTA model.			
TASK 3 – Investigate combined immunomodulatory and neuroregenerative effect of systemic MSC therapy in an allogeneic CTA model			
MILESTONE 3 – Complete allogenic hindlimb transplants		Group VI (allogenic hindlimb transplant) Group VII (allogenic hindlimb transplant + high-dose FK506) Group VIII (allogenic hindlimb transplant + MSC) Group IX (allogenic hindlimb transplant + MSC + high-dose FK506) Group X (allogenic hindlimb transplant + MSC + reduced-doseFK506)	
DELIVERABLE 3 – Improved immunomodulation and nerve regeneration in an allogeneic CTA model		Sequential Catwalk, electrophysiology and histomorphometry Immune monitoring by flow cytometry analysis and MLR assays	

As part of implemented transition plan from University of Pittsburgh to Johns Hopkins University, a state of the art CTA lab was established at Johns Hopkins University with purchase of new equipment and recruitment of new research fellows and technicians. In order to optimize the new equipment and techniques, there was significant delay in performing the proposed experiments as outlined in original Statement of Work (SOW). As shown, we have now completed optimization of cell culturing protocols, surgical techniques, electromyography, functional movement assessment, and nerve histomorphometry. We have completed all animal surgeries for task 1 and are in the process of collecting the remaining functional outcome data for the experiment. In addition, we are now moving forward with the necessary surgeries for tasks 2 and 3. We have revised our SOW to accomplish our remaining tasks and to achieve our milestones in a timely fashion. Therefore, we request additional six months at no extra cost to be granted to accomplish our goals.